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HETEROGENOUS PROTEINS IN KERATOHYALINE GRANULES STUDIED BY QUANTITATIVE AUTORADIOGRAPHY

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Electron microscopic autoradiography with [^{3}H]histidine, [^{3}H]cystine, [^{3}H]arginine, and [^{3}H]proline was used to study protein synthesis in keratohyaline granules of newborn rats. All ^{3}H-amino acids were incorporated into proteins in the granular cells, and the radioactive proteins appeared in the keratohyaline granules. However, the amount of radioactivity associated with the granules and the pattern of ultrastructural localization of the radioactive proteins differed considerably for each ^{3}H-amino acid. "Histidine-labeled" protein was located mainly in the matrix portion of keratohyaline granules whereas "cystine-labeled" protein accumulated in the dense homogenous deposits. "Arginine-labeled" protein was distributed more diffusely in the organelles of granular cells, but that associated with keratohyaline granules seemed to localize mostly with "histidine-labeled" protein and partly with "cystine-labeled" protein. Large amounts of "proline-labeled" protein were also present in other areas of the cytoplasm than keratohyaline granules. This protein localized in the dense homogeneous deposits, but it seemed to turn over more rapidly than "cystine-labeled" protein, an indication that the dense homogenous deposits consist of at least two different polypeptide chains, one of which contains higher cystine and the other higher proline.

Autoradiographic studies have defined the primary sites of incorporation of various injected tritiated amino acids in the epidermis [1–4]. Grain counts showed that the ratio of labeling was higher in granular cells than in basal cells after the injection of [^{3}H]histidine, [^{3}H]arginine, and [^{3}H]serine and lower after the injection of [^{3}H]leucine, [^{3}H]-lysine, and [^{3}H]valine. Labeling was equal in basal and granular cells after the injection of [^{3}H]proline and [^{3}H]tyrosine. Separate studies showed that the incorporation of [^{3}H]cystine was also greater in granular cells than in other parts of the epidermis [5]. Ultrastructural studies, however, indicated that the labeling sites in granular cells were different for [^{3}H]histidine and [^{3}H]cystine. After the injection of [^{3}H]histidine, most of the silver grains first appeared outside the keratohyaline granules and 6 hr later were concentrated inside the granules [6]. [^{3}H]Cystine, on the other hand, along with dense homogenous deposits, appeared to be localized at the edge of the keratohyaline granules [7].

We have demonstrated that keratohyaline granules are composed of cytochemically heterogenous constituents [8]. One of them, the cystine-containing component, may be the protein isolated by Matoltsy and Matoltsy [9] and another, the histidine-containing component, may be the protein studied by Hoober and Bernstein [10], Sugawara and Bernstein [11], and Sibrack, Gray, and Bernstein [12].

In this study we injected [^{3}H]histidine, [^{3}H]-arginine, [^{3}H]proline, and [^{3}H]cystine into newborn rats and used electron microscopic autoradiography for quantitative analysis. In addition, we examined the effects of puromycin on the incorporation of [^{3}H]histidine and [^{3}H]cystine to ensure that the amino acids were actually incorporated into protein.

MATERIALS AND METHODS

Effect of Puromycin on [^{3}H]Cystine Incorporation into Epidermal Protein

Puromycin (Nutritional Biochemical Co.), diluted with saline to concentrations of 0.5 and 1 mg/0.1 ml, was injected intradermally into the right dorsal side of two groups of 6 newborn rats each [13]. The left sides of the same animals were injected with saline as controls. One hour after the puromycin, half of the animals were injected intraperitoneally with 10 μc of [^{3}H]histidine and the other half with 10 μc of [^{3}H]cystine.

One hour later skin samples were obtained from the puromycin-injected and saline-injected sites and were prepared by the following method, which eliminates free ^{3}H-amino acids from the epidermis [6]. The epidermis was separated from the dermis by soaking for 10 to 15 min at 0°C in 0.24 M NH_4Cl, pH 9.5. The epidermis was fixed at 4°C for 12 hr in 3% phosphate-buffered glutaraldehyde and washed in the phosphate buffer for 3 hr. The tissue was dehydrated in absolute methanol, dried in a desiccator, and weighed. It was hydrolyzed in hot 6 N HCl, and the radioactivity was analyzed in PPO–triton–toluene solution (PPO 6 gm/L in Triton X:toluene 1:2 mixture) in a scintillation spectrometer (Beckman LS-150). The rates

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of a protein synthesis in the experimental skin were expressed as a percentage of the DPM/mg tissue of the control skin.

Autoradiography

Twenty microcuries of L-[^{3}H]histidine (4 Ci/mmole, generally labeled), L-[^{3}H]cystine (0.8 Ci/mmole, generally labeled), L-[^{3}H]arginine (5 Ci/mmole, generally labeled), or L-[^{3}H]proline (3 Ci/mmole, generally labeled) in 0.1 ml saline were injected intradermally in the dorsal side of 24 4- to 5-day-old rats (Sprague–Dawley strain). Skin specimens were obtained from the injected sites at 1 and 6 hr after injection. The tissues were cut into small pieces with razor blades, fixed in 3% glutaraldehyde, and postfixed in 2% osmium tetroxide. Both fixatives were buffered with phosphate. The tissues were then embedded in a mixture of plastic, cut at 600 to 800 Å, and placed on stainless steel grids coated with Formvar and carbon. Three pieces were randomly selected from each animal and 6 to 8 sections were cut from each tissue. The grids were filmed with Ilford L4 emulsion by a loop method described by Caro and vanTubergen [14]. After a 9-week exposure at 4°C, the specimens were developed, fixed, and stained with uranyl acetate and lead citrate. Specimens showing more than 2 grains per field over cornified cells were considered to have a high background and were omitted from the data.

Quantitative Analysis

Photographs (× 4000) of all granular cells in the two outermost layers were enlarged by 3 times. The number of silver grains over keratohyaline granules and other areas of the cytoplasm were counted (separate grain counts were made in the nuclei but the results will not be included in this report). The total area studied and the total number of grains counted per animal ranged between 12,000 and 18,000 sq micron between 250 and 700, respectively. At various time intervals after isotope injection, grain counts were analyzed by two different methods. Since the section thickness, radioactive source, and condition of emulsion satisfied the requirements outlined by Salpeter, Beckman, and Salpeter [15], we used their figure of 160 nm for the radius to determine tritium source points.

Percent label of keratohyaline granules. We made the first analysis by counting the number of grains located over the keratohyaline granules and the rest of the cytoplasm of granular cells. The location of grains in keratohyaline granules was further divided into three parts: the dense homogenous deposits (DHD), the rest of the edge, and the center. The number of grains in each compartment was calculated as the percent of the total grain counts.

Specific grain concentration over keratohyaline granules. We made the second analysis by computing the specific concentration of grains over each subcompartment. To do this, we determined the percentage of area occupied by the total keratohyaline granules, as well as the DHD, edge, and center of keratohyaline granules, by superimposing a lattice with regularly spaced points over the electron micrograms [7]. The results obtained by previous study had established that keratohyaline granules occupy 15% of the total granular cell area, DHD 0.98%, and other areas of the edge and center of the granules 1.7% and 12.2%, respectively. The specific grain concentration was obtained by dividing the number of silver grains over each subcompartment by these figures.

RESULTS

Protein synthesis is measured by the incorporation of [^{3}H]cystine into the epidermis 1 hr after injection of puromycin is inhibited. Puromycin (0.5 mg) inhibits histidine incorporation 90% and cystine incorporation 78%. Puromycin (1.0 mg) leads to an 87% inhibition of histidine incorporation and an 88% inhibition of cystine incorporation.

As in a previous study [2], [^{3}H]histidine, [^{3}H]arginine, [^{3}H]proline, and [^{3}H]cystine were incorporated by the granular cells at 1 hr after injection. The number of grains over keratohyaline granules was highest after the injection of [^{3}H]histidine and [^{3}H]cystine, second highest after [^{3}H]arginine, and lowest after [^{3}H]proline. The number of grains over keratohyaline granules increased at 6 hr after the injection of [^{3}H]histidine and [^{3}H]arginine but considerably less after [^{3}H]proline and much less after [^{3}H]cystine (Tab. I)

The distribution of silver grains over keratohyaline granules was quantitatively analyzed. The position of grains was tabulated according to DHD, other areas of the edge, and the center of the granules. When a circle with a radius of 260 nm, drawn at the midpoint of each silver grain, touched the DHD, we considered it labeled. Grains at other areas of the edge of keratohyaline granules were counted as at the edge. Sometimes we were uncertain whether DHD were present underneath the silver grains. Such grains were considered as at the edge but not DHD. Silver grains located inside the keratohyaline granules and clearly more than 160 nm from the edge of the granules were considered as at the center.

Table I summarizes the percent labeling over subcompartments of granular cells at 1 and 6 hr after the injection of ^{3}H-amino acids. It was highest at the edge of the keratohyaline granules and lowest over DHD at 1 hr after the injection of [^{3}H]histidine and [^{3}H]arginine, but highest over DHD after the injection of [^{3}H]cystine. After [^{3}H]proline injection, half the silver grains appeared at the edge and one-quarter were located over DHD and the center of the granules. At 6 hr after the injection of all amino acids, the number of grains over the center of the granules increased, but the degree of increase varied according to the isotopes: after [^{3}H]histidine, 67%; after [^{3}H]arginine, 53%; after [^{3}H]proline, approximately 30%; and after [^{3}H]cystine, only 15%.

Specific grain counts, on the other hand (Tab. II), showed that [^{3}H]histidine-containing protein concentrated at the edge of the keratohyaline granules and subsequently distributed throughout the granules; at no time, however, did it concentrate in DHD. In contrast, [^{3}H]cystine-containing protein concentrated heavily in DHD. The distribution of silver grains after the injection of [^{3}H]proline and [^{3}H]arginine proved less clear-cut and showed a more even distribution of grains over the subcompartments of keratohyaline granules and outside the granules. Radioactivity from

TABLE I. *Percent label over different subcompartments of granular cells at 1 and 6 hr after the injection of [^3H]histidine, [^3H]cystine, [^3H]arginine, and [^3H]proline*

	Keratohyaline granules			Other area of cytoplasm
	DHD	Edge	Center	
[^{3}H]Histidine				
1 hr	1.17 ± 0.52	24.55 ± 2.9	6.7 ± 2.7	67.4 ± 6.1
6 hr	0.87 ± 0.65	11.0 ± 0.99	67.8 ± 5.8	20.1 ± 4.5
[^{3}H]Cystine				
1 hr	18.2 ± 4.3	9.4 ± 1.8	7.4 ± 0.89	64.6 ± 3.8
6 hr	13.6 ± 3.6	10.6 ± 1.1	15.4 ± 2.2	60.4 ± 3.6
[^{3}H]Arginine				
1 hr	0.9 ± 0.3	17.0 ± 9.9	8.0 ± 5.3	74.0 ± 15.5
6 hr	1.8 ± 0.9	7.6 ± 3.4	53.2 ± 7.6	36.9 ± 7.1
[^{3}H]Proline				
1 hr	4.4 ± 1.1	8.7 ± 1.6	4.4 ± 2.3	82.3 ± 3.7
6 hr	1.1 ± 0.3	14.3 ± 9.4	31.2 ± 6.9	53.2 ± 2.7

TABLE II. *Percent specific grain counts over different subcompartments of granular cells at 1 and 6 hr after the injection of [^3H]histidine, [^3H]cystine, [^3H]arginine, and [^3H]proline*

	Keratohyaline granules			Other area of cytoplasm
	DHD	Edge	Center	
[^{3}H]Histidine				
1 hr	6.88 ± 2.2	85.22 ± 2.0	3.14 ± 0.88	4.73 ± 1.1
6 hr	6.78 ± 4.6	49.26 ± 3.9	42.16 ± 5.0	1.77 ± 0.34
[^{3}H]Cystine				
1 hr	73.44 ± 5.9	20.63 ± 5.8	2.64 ± 0.75	3.27 ± 6.5
6 hr	59.94 ± 5.7	30.87 ± 6.0	6.35 ± 1.1	3.38 ± 0.78
[^{3}H]Arginine				
1 hr	7.89 ± 1.7	78.61 ± 6.6	5.02 ± 0.87	8.48 ± 5.8
6 hr	14.60 ± 5.3	41.05 ± 11.0	40.30 ± 10.2	4.04 ± 1.2
[^{3}H]Proline				
1 hr	41.16 ± 4.8	46.54 ± 3.5	3.24 ± 1.6	9.04 ± 1.9
6 hr	8.28 ± 1.6	62.56 ± 7.8	22.76 ± 13.7	5.38 ± 2.4

[^{3}H]proline, as from [^{3}H]cystine, initially concentrated over DHD but also appeared at the edge. At 6 hr after injection, the concentration over DHD declined and that over the center increased. The grain density in the cytoplasm outside the keratohyaline granules was highest at 1 hr and 6 hr after the injection of [^{3}H]proline but not of the other ^{3}H-amino acids used. Labeling patterns after [^{3}H]arginine injection somewhat simulated those of [^{3}H]histidine, except that the concentration of grains over DHD and the area outside of keratohyaline granules was higher after [^{3}H]arginine than after [^{3}H]histidine.

DISCUSSION

Electron microscopic autoradiography of the incorporation of ^{3}H-amino acids into protein of the epidermis confirmed our previous observation that protein(s) is synthesized in granular cells as well as in basal and spinous cells. The possibility that the silver grains detected by this technique may result from ^{3}H-amino acids injected in the tissue in a form other than that of a peptide-bonding has been considered [16,17]. This problem is particularly relevant when [^{3}H] cystine is used to study protein synthesis, because [^{3}H]cystine might be attached to a side group of preexisting peptides by disulfide linkages. We have already used chemical techniques to solubilize nonprotein-bound radioactivity in trichloroacetic acid, EDTA, and thioglycollate [5,6] and have found that our techniques for preparing the tissues removed almost all radioactivity which was not incorporated de novo into protein. To confirm these chemical studies, we examined the effects of puromycin on protein synthesis with [^{3}H]cystine by a technique similar to that used with [^{3}H]histidine [12]. At 1 hr after the injection of puromycin, protein synthesis was considerably reduced both in the animals injected with [^{3}H]cystine and in those injected with [^{3}H]histidine. Thus in granular cells, injected [^{3}H]cystine, like [^{3}H]histidine, is primarily incorporated into protein by de novo synthesis.

All injected ^{3}H-amino acids appeared in keratohyaline granules, the number of grains increasing at 6 hr after injection. However, the most striking concentration over keratohyaline granules was seen after injection of [^{3}H]histidine. Silver grains

concentrated first at the edge of the granules and then over the center, but a considerably smaller number of grains appeared over DHD. [^{3}H]cystine also showed a heavy concentration of grains over keratohyaline granules. However, the density of grains occurred because of extremely high specific counts over DHD; the number of grains over other parts of the granules was the lowest of the amino acids studied. These findings coincide with cytochemical findings, namely that keratohyaline granules consist of heterogenous components. These findings demonstrate the localization of proteins, one of which has a high ratio and another a very low ratio of histidine to cystine. Furthermore, the observations suggest that subcompartments of keratohyaline granules differ from each other not only ultrastructurally but also chemically. The results may indicate that the protein isolated in Matoltsy's laboratory is a constituent of DHD and the protein studied by Bernstein's group comes from another area. The technique used by Matoltsy's group seems to favor collection of DHD and may prove useful in further studies of this complex and poorly understood component of granular cells. On the other hand, the technique used by Sibrack, Gray, and Bernstein [12] and originally introduced by Ugel [18] apparently solubilized a protein which represents another component of keratohyaline granules and is relatively rich in histidine.

The ultrastructural localization of proteins, in which [^{3}H]proline and [^{3}H]arginine were incorporated, still remains speculative. [^{3}H]Arginine appears to incorporate into the histidine protein because grains over the center of the granules increase with time after [^{3}H]histidine injection as well as after [^{3}H]histidine injection. Biochemical studies which indicate that histidine protein contains large amounts of arginine lend additional support to this probability. The finding that {^{3}H]arginine also appears in DHD suggests that this amino acid is also present in proteins of DHD. The fact that grains counted over the cytoplasm were considerably more prevalent than those counted after [^{3}H]histidine injection indicates that [^{3}H]arginine also was incorporated into protein(s) in the cytoplasm which was not added to keratohyaline granules. Moreover, our previous study has shown that arginine incorporates into protein(s) of the nuclei of differentiated cells [19]. Thus, this amino acid seems to be involved in many aspects of epidermal cell differentiation and is not, therefore, a good marker in studies dealing with a specific component of granular cells.

The large amounts of [^{3}H]proline located over DHD at 1 hr after injection strongly suggest that this amino acid is a constituent of protein in DHD. According to the biochemical analysis of Matoltsy and Matoltsy [9], the protein of keratohyaline granules contains high cystine and proline. The present study appears to confirm this and to indicate that there is a protein which contains large amounts of cystine and proline. However, the decline in grain counts in DHD at 6 hr after the injection of proline was much greater than that after the injection of [^{3}H]cystine; thus at least two polypeptides with different turnover rates may be present in DHD. A biochemical study which uses [^{3}H]cystine and [^{3}H]proline to isolate pure protein fractions from DHD may help to explain the autoradiographic observation.

Recently MacCallum and Han used electron microscopic autoradiography to study the incorporation of [^{3}H]proline into mouse lingual epithelium [20]. They found that [^{3}H]proline incorporated into keratohyaline granules but that "only one silver grain appeared over each granule." In the present study, we also found that large amounts of [^{3}H]proline incorporated into other cytoplasm besides keratohyaline granules and that even without calculating specific grain concentration, the grain counts did not demonstrate the specific association of grains over DHD.

The present study also indicates that electron microscopic autoradiography is useful for relating biochemical information to the organelles and structures found in granular cells. The information obtained seems to explain the conflicting biochemical results obtained by other investigators in this field. An awareness that keratohyaline granules are composed of several chemically heterogenous components provides a basis for understanding the morphologic variations of this structure in normal and pathologic granular cells. We regard any changes in the amount of these different components of keratohyaline granules as being directly related to the morphology of granular cells. Thus, biochemical studies designed to characterize each pure component of keratohyaline granules appear to be useful for comprehending the function of granular cells in pathologic states.

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